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A protein molecule as a bioanalytical device

Chemomechanical protein sensors

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Cross-linked protein solids are proposed as new types of chemomechanical sensors for identification of biological molecules and measurement of their concentration. New sensors make use of the ability of proteins to specifically bind its ligands and to alter their conformation upon the binding. The use of immobilized proteins enables these conformational changes to be detected through the changes in mechanical properties of protein solid samples (stress, stain or the Young's modulus). Some advantages of the new sensors over enzyme probes are illustrated on considering the properties of papain sensor for α-*N*-benzoyl-L-arginine ethyl ester.

Immobilized enzyme *Binding assay* *Papain* *Elastic response* *Protein sensor*

1. INTRODUCTION

The increasing demand of biochemical and clinical research, control of biotechnological industrial processes and monitoring of environmental pollution generates a need for sensors that enable specific detection and measurement of concentration of definite substances in some complex media. Most difficulties arise in analysis of biochemical substances where laborious, time-consuming and expensive separation and identification procedures are often required.

A number of specific sensors for the analysis of biological compounds (enzyme electrodes and other immobilized enzyme probes) have been developed recently [1,2] following the principle proposed in [3]. The immobilized enzyme probe is a combination of a thin layer of immobilized enzyme with any type of sensor capable of giving information of local changes in the properties of a medium that occur during the enzyme reaction in the region where the immobilized enzyme is placed. The presence of a suitable enzyme reaction and convenient method for monitoring the local transient changes in the medium, the necessity for a second substrate, stringent requirements for enzyme purity and some other factors following the general principle of their construction (direct electrochemical interference with ion-selective electrodes, influence of stirring etc.) considerably limit the number of substances the enzyme probe can be made for [2].

It was concluded from our recent studies on solid protein samples that their mechanical properties were highly sensitive to binding specific ligands [4,5]. This result enabled us to advance some new principles concerning the designing of the specific sensors based on chemomechanical interactions in proteins.

2. NEW PRINCIPLES OF PROTEIN SENSOR DESIGNING

Firstly, the changes in protein conformation and other properties, resulting from the specific binding of the substance under analysis are suggested to be used for detecting and measuring the concentration of the substance. Many proteins (enzymes, antibodies, proteins for storage and transport of biological molecules) have been shown to change their conformation upon binding the specific ligands. The combination of such a protein with a

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physical device capable of detecting this conformational change may be considered as a new type of protein specific sensor. Apart from some obvious advantages of immobilized proteins over dissolved ones (enhanced stability, lack of protein loss in analysis) the cross-linked protein crystals and amorphous solids enable the conformational changes in proteins to be detected with a sensitivity superior to any known physical method in solution. To detect conformational changes in proteins through changes in the mechanical properties of its cross-linked solids is our second proposal.

Fig.1 illustrates the operation of the sensor and the origin of high sensitivity of mechanical measurements using protein cross-linked samples. Let the average characteristic length of the protein l be changed to $(l - \Delta l)$ as a result of a ligand binding. The relative change of macroscopic characteristic length of the sample L must be obviously equal to that of l, $(\Delta l/l) = (\Delta L/L)$, provided the protein molecules make a lattice through their intermolecular contacts or short cross-links. A relative change of the macroscopic length L as small as 10^{-6} can be easily detected. For a protein molecule 40 Å in size this means sensitivity to 4×10^{-5} Å change in characteristic length l. This value should be considered as a molecular characteristic averaged on a great assembly of protein molecules and over a large period of measurement that considerably exceeds the characteristic times of molecular movement.

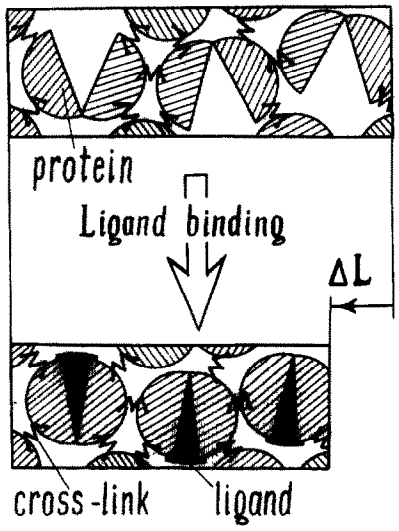

Fig.1. Principle of sensor operation. Schematic presentation of the change in length ΔL of the cross-linked protein solid upon specific ligand binding.

Apart from ligand induced strain of the sample the changes in a protein and sample rigidity can give information about the ligand binding. Our recent study of inhibitor binding to hen egg-white lysozyme has shown considerable increase in the rigidity of its solid samples displaying the immobilization of lysozyme domains upon inhibitor binding in the active site cleft [4,5].

We present here an example of the experimental realization of the principles discussed above for constructing a sensor for α-*N*-benzoyl-L-arginine ethyl ester (BAEE) using its ability to specifically bind to papain, changing its overall conformation.

3. EXPERIMENTAL

An amorphous film of mercury-papain (Fluka) was prepared from its water solution (200 mg/ml), poured out onto a glass plate in a thin layer and rapidly dried at reduced atmospheric pressure on evacuation (10–15 mmHg). Dry protein film (5–10 μm thick) was cross-linked in a vapor of 25% glutaraldehyde solution for 6 h at room temperature [6]. To remove the glutaraldehyde the cross-linked film was washed with water and carefully detached from the glass plate, dried and stored in a refrigerator. Before the measurement a strip 500 × 50 μm was cut from a place of the film placed in a plastic cell filled with water. The strip was then attached to two tungsten micropincettes similar to that described in [6]. One of the micropincettes was attached to a force transducer, another being attached fixedly. The force transducer was designed as a capacitor transducer made from a fused quartz with a vacuum deposited gold coating. The bending of the quartz plate under the action of the force applied resulted in a change in the capacitance connected into a circuit of a capacitometer. The strip was submerged into a cell 30 μl in volume filled with buffer or solution under study. A more detailed description of the experimental installation and handling procedures will be given elsewhere.

4. RESULTS AND DISCUSSION

The papain strip was stretched up to 4.5% of its initial length and allowed to relax for half an hour

in a buffer solution. The buffer was then exchanged for a series of standard BAEE solutions in the buffer. On binding BAEE papain strip tends to shorten causing the isometric stress to increase. The response of the sensor to various BAEE concentrations may be seen from fig.2 to be linear to about 10–15 mM. Double reciprocal plot analysis shows this curve to be an adsorption isotherm described by two dissociation constants: K_1 = 20 mM and K_2 = 60 mM. The former is close to that for BAEE binding to papain in solution [7]. BAEE binding and sensor response are fully reversible. The response time of the sensor is mainly determined by diffusion of the ligand through the protein film. It takes 1–2 min to reach equilibrium binding in 7–10 μm thick film. This enables the sensor to be used for monitoring the BAEE concentration changes in a trypsin catalysed reaction (fig.3).

The mercury–papain chemomechanical sensor clearly illustrates some advantages of the new type of sensors over enzyme probes. For example, should the sensor for BAEE be made as a combination of enzymatically active papain film and pH glass electrode it would be far less stable. Films of papain lost their BAEE binding ability in our experiments for 1–3 h due to the autolysis. Mercury modification of the cysteine in the active site of the enzyme greatly enhanced the stability (at least up to several days) due to complete suppression of the enzyme reaction and autolysis retaining its ability to specifically bind the substrate and to change the protein conformation. It also means that besides the enhanced stability the sensor is capable of working in media contaminated with heavy metal ions, poisoning the enzyme electrodes. The lack of the destruction of the substance analysed may also be of importance in some cases. The most promising advantage of the sensors appears to be the great variety of proteins which can be used in its construction. We can think of no reason why the sensor could not be used for any biological or biologically active substance.

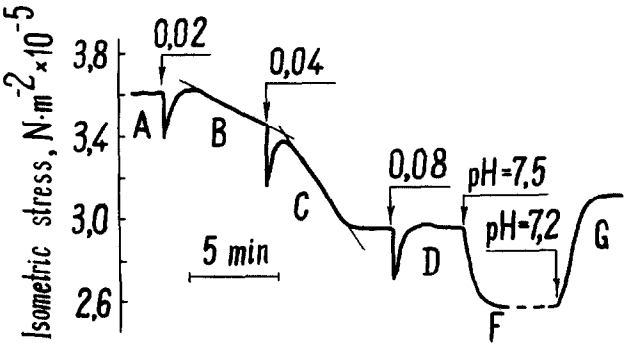

Fig.3. An example of the mercury papain sensor use for recording the trypsin hydrolysis of BAEE. A, the sensor is placed in solution of 10 mM BAEE, 0.1 M K-phosphate, pH 7.5. B, C and D, trypsin solution in the buffer (2 mg/ml) was added to final concentrations denoted by figures above the arrows. F, the hydrolyzate (pH 7.2) was exchanged for the 0.1 M K-phosphate buffer (pH 7.5). G, the buffer of pH 7.5 was exchanged for the same buffer of pH 7.2.

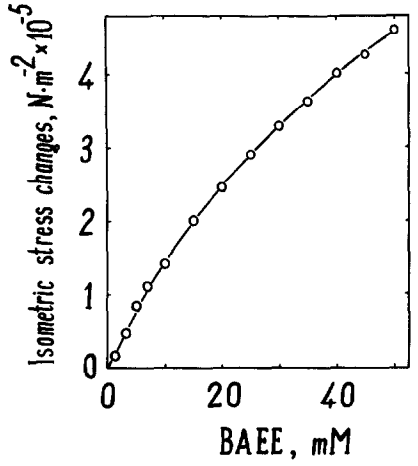

Fig.2. Response of mercury-papain sensor to different-concentrations of BAEE. Initial isometric stress in 0.1 M K-phosphate buffer (pH 7.5) was 3.3 × 10^5 N·m^{-2}.

We have tested a number of proteins for the ability to be used as a material for the sensors. Carp parvalbumin, yeast hexokinase, hen egg-white lysozyme [4,5], acetylcholinesterase from human erythrocytes and some other protein films responded specifically on submerging them into solutions of their specific ligands at physiological concentrations. The data will be published elsewhere.

Chemomechanical sensors can be designed in such a way as to work in the absence of any measuring device. Should the sensor be made double-layered the small length changes upon ligand binding in one layer would transform into considerable lateral binding, as occurs in bimetallic

plates used for temperature measurement. For example, both layers of the bimorph could be made from the same protein, the active sites of the protein in one layer being prevented from ligand binding by affinity labelling. Such a bimorph differential transducer appears to be very attractive and quite promising for it would be free of pH, ionic strength, temperature and other unspecific interference which would affect chemomechanical sensors. Consisting of only pointer and scale this analytical device could become the most simple and inexpensive for routine biochemical and clinical analysis. Our preliminary experiments with Hg- and Hg fluorescein–papain double-layers proved these differential sensors to be feasible.

5. CONCLUDING REMARKS

The approach used here to make the sensors may also have extensive application in the rapid testing of proteins for their ability to bind various substances and to change their conformation upon binding. Apart from purely scientific interest and application in the study of very fine conformational changes, such a study will be useful in pharmacology for a rapid screening of the substances to find those which bind and alter the protein conformation. The samples for the study would require only 10^{-3} mg of protein.

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